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CRISPR/Cas9-based genome editing for simultaneous interference with gene expression and protein stability

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ABSTRACT

Interference with genes is the foundation of reverse genetics and is key to manipulation of living cells for biomedical and biotechnological applications. However, classical genetic knockout and transcriptional knockdown technologies have different drawbacks and offer no control over existing protein levels. Here, we describe an efficient genome editing approach that affects specific protein abundances by changing the rates of both RNA synthesis and protein degradation, based on the two cross-kingdom control mechanisms CRISPRi and the N-end rule for protein stability. In addition, our approach demonstrates that CRISPRi efficiency is dependent on endogenous gene expression levels. The method has broad applications in e.g. study of essential genes and antibiotics discovery.

INTRODUCTION

The combined impact of synthesis and degradation dynamically determines protein levels in living cells. There is a growing need for synthetic biology tools that can control the abundance of specific proteins, e.g. for the fine-tuning of enzymes in metabolic pathways or studies of essential genes for which genetic knockouts are lethal (1). Existing methods typically focus either on genetic knockouts, conditional repression of transcription or direct interference with protein function or stability. However, a combination of these approaches is desirable to achieve a more controllable, rapid or stronger repression of the amount of selected proteins in the cell. Furthermore, for conditional removal of proteins, stability is a key factor even in fast-growing microbes such as yeast where the majority of proteins are very long-lived (2).

Given the recent rapid development in synthetic biology and genome editing technologies, we asked to what extent it was possible to harness generic molecular mechanisms for

simultaneously controlling both protein synthesis and stability. To this end we first looked for a cross-kingdom protein regulatory mechanism. The N-end rule states that the identity of the N-terminal residue (N-degron) of a protein is a prime determinant of its half-life across all kingdoms of life (3). Conveniently, the tobacco etch virus (TEV) protease can accommodate most amino acid residues in the P1' position following the cleavage site (ENLYFQ↓X, where X denotes all amino acids except proline) and thus this small recognition site can mask an N-degron (4,5), and we noted that the N-terminal location enables simultaneous manipulation of the translational initiation region (TIR) - a region surrounding the start codon that has a major impact on gene expression levels (6,7). This enables manipulation of both protein degradation and translation initiation, which is important when manipulating essential genes as shown later.

The CRISPR–Cas9 system enables cutting of very specific DNA sequences in a wide variety of living organisms and has revolutionized our ability to edit genetic information (8). The system has also been repurposed to regulate transcription—e.g. to activate (9) or repress gene expression in e.g. *Escherichia coli* (10), *Bacillus subtilis* (11), human cells (12) and *Saccharomyces cerevisiae* (13) in a general approach known as CRISPR interference (CRISPRi). CRISPRi is based on a catalytically inactive Cas9 endonuclease (dCas9), which can interfere with transcription by binding to specific DNA sequences with the aid of a guide RNA. However, the efficiency of CRISPRi-based systems is affected by endogenous gene expression levels (9). Thus, concomitant manipulation of endogenous gene expression levels may expand the applicability of CRISPRi. Here, we develop a single workflow that combines conditional protein degradation with CRISPRi and TIR manipulation and apply the system to study essential genes and develop strains hypersensitive to antibiotics.

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MATERIALS AND METHODS

Plasmid construction

The pPROTi plasmid resulted from a triple PCR-fragment assembly *via* USER cloning, as described previously (14). For further details on all the plasmids described here see Supplementary Table S1. We amplified the pSEVA33 backbone, the L-rhamnose inducible promoter PrhaBAD, and the S219D mutant of TEV protease from pSEVA331 (15), pKS1 (16) and pKM586 (17) with the oligonucleotides oMSB1270/1267, oMSB1268/1269 and oMSB1271/1273, respectively. For further details on all the oligonucleotides described here see Supplementary Table S2. The pMAZ-SK plasmid with different guide RNAs used to PROTi tag essential genes by CRMAGE were constructed by USER cloning (18). This was done by mixing two annealed oligonucleotides that were complement to the amplified pMAZ-SK backbone after USER treatment, as described previously (19). Specifically, for the genes *accD*, *fabG*, *ftsZ*, *glmS*, *ileS*, *murE*, *pheS*, *ribD*, *prfB*, *rnpA*, *tmk*, *acpS*, *ispH*, *murA*, *dapE*, *lpxC* and *ribE*, the oligonucleotide pairs oMSB2565/2566, oMSB2591/2592, oMSB2569/2570, oMSB2571/2572, oMSB2573/2574, oMSB2575/2576, oMSB2577/2578, oMSB2593/2594, oMSB2579/2580, oMSB2583/2584, oMSB2585/2586, oMSB2750/2551, oMSB2740/2741, oMSB2742/2743, oMSB2748/2749, oMSB2744/2745 and oMSB2746/2747 were used, respectively. To construct the pCRiPi plasmid, the pPROTi plasmid was PCR amplified using the oligonucleotide pair oMSB1865/2312, and the *dCas9* gene (including the aTc promoter and the terminator) was amplified from pdCas9 with the oligonucleotides oMSB2313/oMSB1866. For construction of the pgRNA-CRiPi plasmid targeting the PROTi tag for the CRiPi system, the pSLQ1236 (Supplementary Table S1) was used as backbone. The gRNA was changed by standard Gibson assembly (20) with oligonucleotides oSONG145/146. Nucleotide sequences of pPROTi, pCRiPi and pgRNA-CRiPi are provided in the supplementary information.

Bacterial strains, media and growth conditions

The parental strain for all experiments was *E. coli* MG1655 (ATCC 47076). *E. coli* MG1655 with GFP integrated into the genome (21) was initially used to tag *gfp* first by recombineering (22) with the ssDNA MAGE oligonucleotide oMSB1277 and then with the ssDNA MAGE oligonucleotides oMSB1275 (unstable GFP variant with phenylalanine as the N-degron) or oMSB1276 (stable GFP variant with alanine). These two GFP variants were used as PCR templates to add the IPTG inducible T5 promoter with the oligonucleotides oMSB1661 and oMSB1662. After, T5-GFP variants were integrated into the *E. coli* MG1655 genome by clonetegration (23), using pOSIP-KT, and the oligonucleotides oMSB1297 and oMSB1298. FLP-mediated excision was performed as previously described (23). All *E. coli* strains were grown in lysogeny broth (LB) at 37°C shaking at 300 rpm. The antibiotics ampicillin (100 µg/mL), chloramphenicol (30 µg/mL) or kanamycin (50 µg/mL) were added when needed. *gfp* expression was induced with 0.1 mM IPTG; *tev* protease expression was in-

duced with 5 mM rhamnose; and *dCas9* endonuclease expression was induced with 200 ng/mL aTc.

Genomic integration of the PROTi tag

The PROTi tag was inserted after the first codon downstream of the start codon in genes of interest, according to the previously described CRMAGE protocol (19). The starting strain for CRMAGE was MG1655 K-12 harboring the pMA7CR_2.0 and pZS4Int-tetR plasmids. The pMA7CR_2.0 plasmid expresses the Cas9 nuclease, the λ-red β-proteins and the dam protein that represses the mismatch DNA repair system for obtaining higher genome editing efficiency (19). 5 µM of ssDNA CRMAGE oligonucleotide and 250 ng of pMAZ-SK plasmid with inserted gRNA were used for electroporation. Cultures were grown at 37°C in Julabo SW22 linear-shaking water-bath at 250 rpm. After aTc addition to express *Cas9* according to the previously published protocol (19), recovery was performed overnight. The ssDNA CRMAGE oligonucleotide contained 35–45 nucleotide end homology on each side of the insertion. For each gene, a PAM sequence (5'-NGG-3') and the adjacent gRNA (20 nucleotides) were chosen in the coding sequence. The pMAZ-SK plasmids with inserted gRNA were constructed as described above. One nucleotide in the PAM sequence was changed in the ssDNA CRMAGE oligonucleotide to avoid Cas9 recognition of mutants with the inserted PROTi tag. Randomization of the TIR was done by changing the specific nucleotides in the ssDNA CRMAGE oligonucleotide used for insertion of the PROTi tag. For the genes *accD*, *fabG*, *ftsZ*, *glmS*, *ileS*, *murE*, *pheS*, *ribD*, *prfB*, *rnpA*, *tmk*, *acpS*, *ispH*, *murA*, *dapE*, *lpxC* and *ribE*, the ssDNA CRMAGE oligonucleotides oMSB2595, oMSB2596, oMSB2597, oMSB2598, oMSB2599, oMSB2600, oMSB2601, oMSB2651, oMSB2602, oMSB2603, oMSB2604, oMSB2757, oMSB2752, oMSB2753, oMSB2756, oMSB2754 were used, respectively.

PROTi characterization

E. coli MG1655 strains containing the PROTi tagged *gfp* in the genome and harboring the pPROTi plasmid, were inoculated from an overnight culture to OD₆₀₀ 0.01 in LB supplemented with chloramphenicol and IPTG. After 4 h of growing, the cultures were induced by adding 5 mM rhamnose. To wash out IPTG from the culture medium before inducing with rhamnose, the cultures were centrifuged and resuspended in the same volume of LB with chloramphenicol and rhamnose. After 1–4 h, cultures were analyzed in a SynergyMx Microplate reader from Biotek. For GFP fluorescence quantification, emission was detected at 512 nm with the excitation light of 480 nm and 80 level gain.

Flow cytometry

Flow cytometry measurements were performed on a FACS Aria (Becton–Dickinson, San Jose, USA) with 488 nm excitation from a blue solid-state laser. Cells were diluted 1:100 in PBS for analysis. At least 20 000 cells were collected for each measurement. FlowJo (Treestar) was used for data analysis.

Growth profiles

Growth was monitored in the BioLector® from m2p-labs. Each of the tagged PROTi clones were diluted 1:100 in 1 ml LB supplied with appropriate antibiotics in a Flower-Plate (48-well MTP, flower) for the BioLector®. Cultures were induced with rhamnose and aTc from the beginning of growth and grown at 37°C, shaking at 1200 rpm.

CFU assays and drop tests

To determine colony forming units (CFU), cultures of PROTi tagged essential genes harboring the pPROTi plasmid were inoculated in 1 ml LB supplied with chloramphenicol and rhamnose in a 96-well microtiter plate. After 4 h of growth, cultures were plated on LB agar plates in serial dilutions. For the CRiPi system, cultures were inoculated in 1 ml LB supplied with chloramphenicol and ampicillin and grown for 4 h. The CRiPi system was induced with rhamnose and aTc and the cultures were grown for additional 4 h before plating. For drop tests, overnight cultures of tagged essential gene variants were diluted to the same OD and 10-fold serial dilutions were performed. From each dilution, 10 µl were placed on LB agar plates with appropriate antibiotics.

Fosfomycin sensitivity

Fosfomycin sensitivity was determined with minimum inhibitory concentration (MIC) assays as previously described (24). Stock solution of fosfomycin disodium (Sigma Aldrich) salt was dissolved in MiliQ water (1 mg/ml). Briefly, 2-fold serial dilutions of antibiotic stocks (from 0.015 to 256 g/l) were made in 150 µl LB medium supplemented with the appropriate antibiotics, rhamnose and aTc (for CRiPi-induction) and OD after 18 h was determined and plotted relative to the growth of the same cells in the absence of fosfomycin. For testing fosfomycin sensitivity upon PROTi induction, cultures of *murE1*-tagged cells harbouring the CRiPi system were inoculated in 1 ml LB supplied with chloramphenicol, ampicillin and rhamnose in a 96-well microtiter plate. After 4 h of growth, cultures were plated on LB agar plates supplied with different concentrations of fosfomycin.

RESULTS

We first engineered a pro-N-degron module by incorporating the corresponding nucleotide sequence at the 5' end of a gene on the *E. coli* chromosome using CRMAGE genome editing (19). The pro-N-degron module consists of a seven amino acid peptide recognition site of the tobacco etch virus (TEV) protease (ENLYFQ↓F) (5) and an eleven amino acid-linker (25). In the presence of TEV protease, the peptide is cleaved and an N-end-rule substrate is generated with phenylalanine as the prime protein-destabilizing factor (Figure 1A). Importantly, the entire coding sequence for the pro-N-degron module is small enough to fit into a standard oligonucleotide compatible with CRMAGE. With this protein interference (PROTi) system, by rhamnose-inducible expression of the TEV protease from a plasmid, the N-degron becomes de-protected and the protein is targeted for proteasomal degradation through the N-end rule pathway.

To characterize the system, the PROTi tag was fused to the N-terminus of GFP by integrating the coding sequence into the *E. coli* genome with a synthetic IPTG-inducible T5 promoter (Figure 1A). In the resulting strain, GFP production was induced by adding IPTG followed by growth for four hours. At this stage, expression of the TEV protease was induced with rhamnose. Three hours after addition of rhamnose, GFP levels showed a strong decrease (83%—measured by whole cell fluorescence) compared to the uninduced control (Figure 1B). Further characterization of the system by flow cytometry revealed a broad population of cells with different fluorescence levels upon induced protein degradation (Figure 1C), which we hypothesized was caused by simultaneous strong GFP synthesis driven by the T5 promoter. Washing out IPTG from the culture medium prior to the induction of the PROTi system with rhamnose confirmed this hypothesis as it resulted in a homogeneous non-fluorescent population (Figure 1D). The reduction in GFP fluorescence can be ascribed to the generated N-degron, since GFP fluorescence remained high in cells harboring a stable PROTi tag variant, with the phenylalanine of the N-degron replaced by an alanine (Figure 1D).

The fact that protein abundance is a function of both synthesis and stability prompted us to turn to the broadly applicable CRISPR–Cas9-based gene regulation technology. With the aim of gaining control over both transcription and protein stability with a single genome-editing step, we developed a CRISPRi–PROTi (CRiPi) system, where dCas9 can be produced together with the TEV protease (Figure 2A), thereby enabling simultaneous inhibition of gene expression as well as degradation of the target protein.

Based on previous studies, the gRNA was designed to bind to the non-template DNA in the 5' end of the gene, and *dCas9* was expressed from a plasmid with an anhydrous tetracycline (aTc)-inducible promoter (10). Moreover, we designed the gRNA so that it only targets the CRMAGE-inserted sequence, which encodes the TEV protease recognition site and the N-degron-linker, thereby creating a generic gRNA target independent from the site of insertion (Figure 2A).

As shown in Figure 2B, cellular depletion of GFP was rapidly achieved with high efficiency when the CRiPi system was induced with both rhamnose and aTc. Specifically, 76% of the cells showed complete loss of fluorescence after two hours of induction. In contrast, the induction of *dCas9* expression alone caused only a slightly reduced GFP fluorescence, denoting the high stability of GFP (Figure 2B).

The technology described above is particularly useful for analysis of genes that are essential for the organism and thus inaccessible with traditional knockout approaches. Thus, to further demonstrate the functionality of the system, we compared the effectiveness of the PROTi, CRISPRi and CRiPi technologies to control the level of proteins encoded by essential genes in *E. coli*. In a previous approach all essential genes in *E. coli* were individually targeted with a sequence encoding a C-terminal protein degradation tag (*mfsrA*) but 67 proteins could not be tagged this way despite repeated attempts (26). We noted that 54 of these 67 'inaccessible' genes were part of operons, making lethal polar effects a likely explanation. Thus, the N-terminal location of

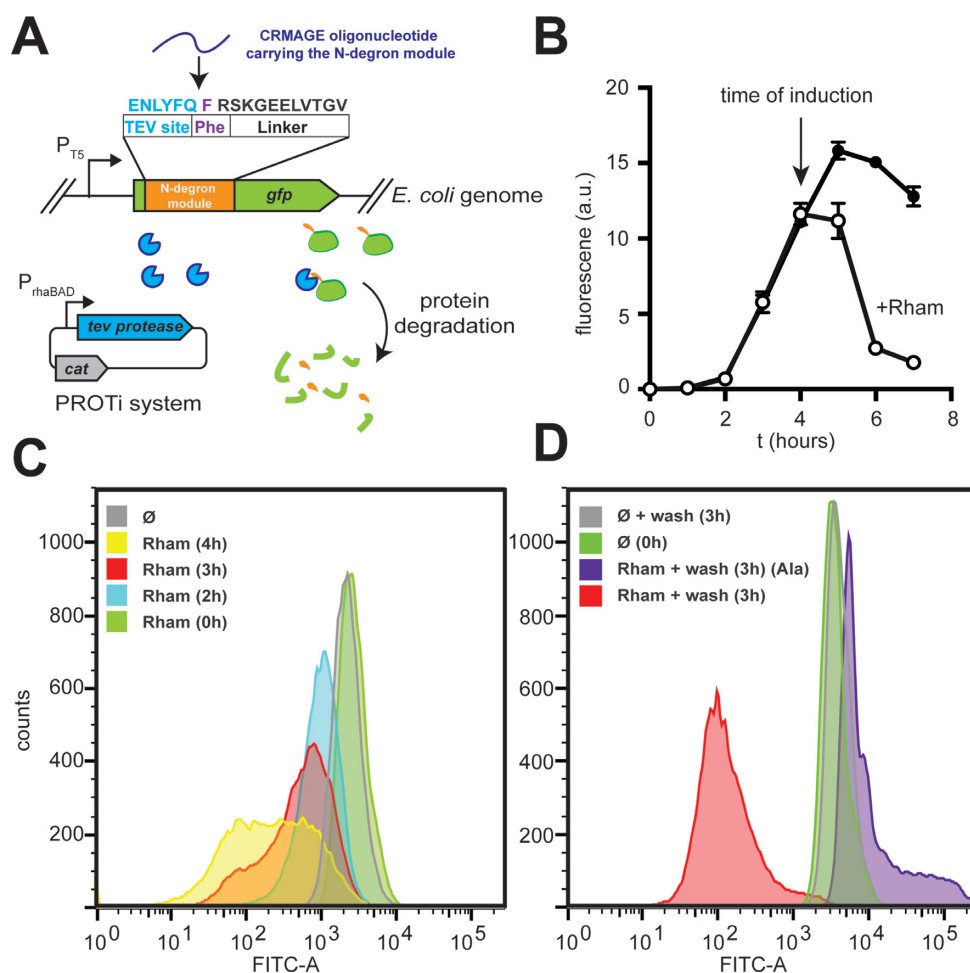


Figure 1. PROTi: Development and characterization of the PROTi system to control protein abundances. (A) Schematic illustration of the PROTi system. The coding sequence for the pro-N-degron module (orange) is integrated by CRMAGE at the 5'-end of a genomically integrated *gfp* (green). *gfp* is under control of the IPTG-inducible T5 promoter. With PROTi, the TEV protease (blue) is expressed from the PrhaBAD promoter leading to de-protection of the N-degron followed by degradation of GFP (green) through the N-end rule pathway. (B) Whole-cell fluorescence measurement of cells expressing GFP tagged with the pro-N-degron—with and without the PrhaBAD inducer rhamnose (Rham). Data represents the average of three biological replicates and bars show the standard error. (C) Flow cytometry histogram displaying the fluorescence signals after 0, 2, 3 or 4 h induction with rhamnose or without induction (Ø). (D) Fluorescence signals after 3 h of PROTi induction with rhamnose, while removing the IPTG inducer from the culture medium by washing. A stable PROTi tag with alanine (Ala) replacing phenylalanine was included as control.

the PROTi tag could enable the targeting of some of these essential genes and since the inserted module overlaps with the TIR, expression tuning by nucleotide variation in this region could minimize polar effects caused by e.g. changes in translational speed.

Using CRMAGE, we attempted targeting of ten essential genes that were not previously *mf-ssrA*-tagged (*glmS*, *ileS*, *murE*, *pheS*, *ribD*, *tmk*, *accD*, *prfB*, *fabG* and *rnpA*) and seven that were previously *mf-ssrA*-tagged (*ftsZ*, *acpS*, *ispH*, *murA*, *dapE*, *lpxC* and *ribE*) (26) using the pro-N-degron module designed with a TIR library made of six random nucleotides upstream from the start codon and all synonymous codons sampled in two positions following the start codon (Figure 3A). Remarkably, this way we were able to identify insertions in seven of the 10 genes that were not previously *mf-ssrA*-tagged, despite their location in essential operons—as well as three out of the seven previously *mf-ssrA*-tagged genes (Supplementary Table S3). Not surprisingly, we also noted a high variability in colony size di-

rectly after CRMAGE, and in cell viability assays (Supplementary Figure S1), as a directly observable consequence of the TIR variation. This is highly useful both for gauging the success rate of CRMAGE and when searching for variants with wild type gene expression levels. For example, for *ileS*, 11 small colonies were screened by colony PCR and all had the PROTi tag inserted. From 12 big colonies, 11 were negative. For the *rnpA* gene, five out of 12 small colonies were positive, whereas 10 out of 11 of the big colonies were not tagged.

To demonstrate the value of the TIR variation approach, for five of the genes (*murE*, *pheS*, *rnpA*, *ileS* and *ribD*), we attempted to insert the corresponding sequences without TIR variation – preserving the six nucleotides upstream from the start codon from the native gene context. For three of these genes we were unable to isolate tagged clones, indicating a lethal effect, whereas we could isolate clones with the *ileS* and *rnpA* genes tagged (Figure 3B and C). However, these clones clearly exhibited growth defects both directly after

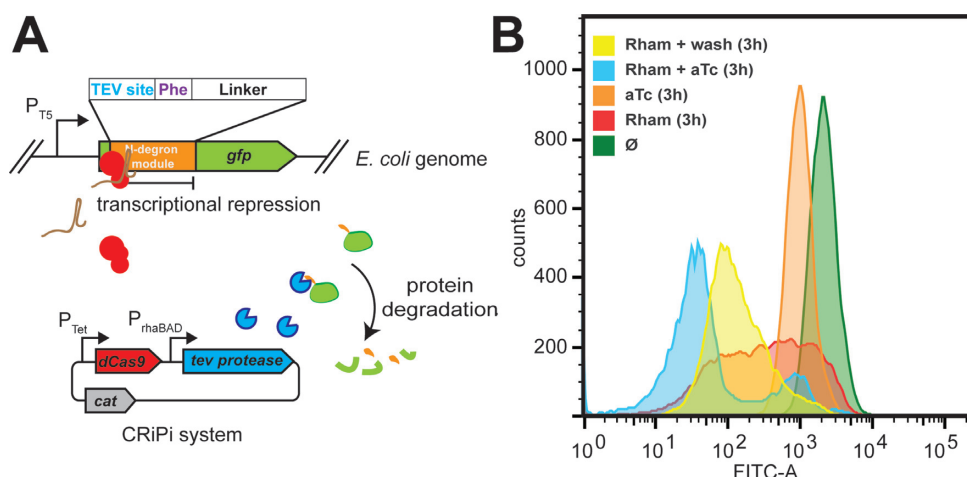


Figure 2. CRiPi: Schematic illustration of the CRISPRi-PROTi (CRiPi) system. (A) Cellular depletion of the targeted protein can be accelerated by simultaneous expression of *dCas9* (red) from the P_{Tet} promoter and the TEV protease (blue) from the P_{rhaBAD} promoter. Here shown for *gfp* as an example. The *dCas9* targets the genomically integrated pro-N-degron encoding sequence with the aid of a guide RNA (gRNA, brown, curved line) and represses transcription. (B) Fluorescence after 3 h of PROTi, PROTi and wash, CRISPRi, or CRiPi induction with rhamnose and the P_{Tet} inducer anhydrous tetracycline (aTc), or with no induction (\emptyset).

CRMAGE and in subsequent viability tests (Figure 3B and C). In contrast, using the TIR randomization approach, we were able to isolate tagged gene variants without any observable growth defects.

After having obtained both growth-affected and unaffected PROTi-tagged gene variants, we moved on to study the CRiPi system, by inducing protein degradation and/or gene silencing. Most of the strains were unaffected in growth in liquid cultures after PROTi induction with rhamnose (Supplementary Figure S2). In fact, rhamnose had a small stimulatory effect on growth in several cases (Supplementary Figure S2). However, the tagged *murE*, *ribD* and *pheS* strains exhibited 1–2 orders of magnitude decrease in viability when plating the cells after PROTi induction (Figure 3D) and most of the strains showed growth retardation after CRISPRi induction with aTc (Supplementary Figure S2). Only the tagged *murE* strain showed a clear effect when inducing PROTi directly in liquid culture (Figure 3E). Of the two approaches utilized in the CRiPi method, CRISPRi had the strongest overall effect (Supplementary Figure S2). However, for the *ileS* and *pheS*-strains the repression of growth after plating was clearly enhanced by simultaneous targeting of both transcription and protein stability (Figure 3F), thereby showing the versatility and strength of the CRiPi method.

To demonstrate the relevance of CRiPi and PROTi for applied biotechnology, we explored its performance as a tool for creating antibiotic hypersensitive strains for use in antibiotic discovery. When screening large compound libraries it is challenging to supply sufficiently high concentrations of each compound, which leads to false negatives in the screen. Lowering the concentration of essential protein targets, enable high-throughput screening with sub-inhibitory drug concentrations and discovery of combinatorial drugs and targets (27). We focused on *MurE*, a central enzyme in peptidoglycan biosynthesis (Figure 3G). When CRiPi or PROTi were induced in cells harboring PROTi-tagged *MurE*, a significant decrease in viability was

observed (Figure 3D and H). We next demonstrated *murE* as a potential target for creating hypersensitive strains that can be used for screening compound libraries to identify agents with antibacterial activity. Fosfomycin is an antibiotic that causes specific inhibition of the enzyme *MurA*, which is involved in the same peptidoglycan biosynthetic pathway as *MurE* (Figure 3G). By applying fosfomycin to cells with an induced CRiPi or PROTi system targeting *MurE*, the sensitivity to the antibiotic increased, depicted as complete growth inhibition at lower concentrations of the antibiotic, compared to the non-induced control (Figure 3I and Supplementary Figure S3). Moreover, fosfomycin sensitivity varied markedly in clones with different *murE*-TIR backgrounds upon induction of CRiPi (Figure 3I and Supplementary Figure S4).

DISCUSSION

Our approach has some limitations: N-terminal peptide tagging of essential proteins may not always be allowed as the tag itself could compromise activity. Furthermore, when attached to some proteins, TEV cleavage or subsequent targeting to the ClpP protease may not be efficient. However, PROTi could serve as a complement to other protein destabilizing technologies (e.g. proteins that are compromised by C-terminal tagging). Here, we were able to target 7 out of 10 proteins that previously had failed with a C-terminal destabilizing tag approach. It is possible that an even higher success rate could be obtained with additional screening efforts (and almost certainly for targeting of non-essential genes). Four out of these seven proteins (encoded by *murE*, *ribD*, *ileS* and *pheS*) were sensitive to induction of PROTi.

CRISPRi generally had the highest growth-effect on essential genes, compared to PROTi, possibly due to the reduced affinity of TEV protease with the N-end rule substrate Phe in the P1' position (5) or because essential protein depletion is compensated for by gene expression upregulation. The approach could potentially benefit from increas-

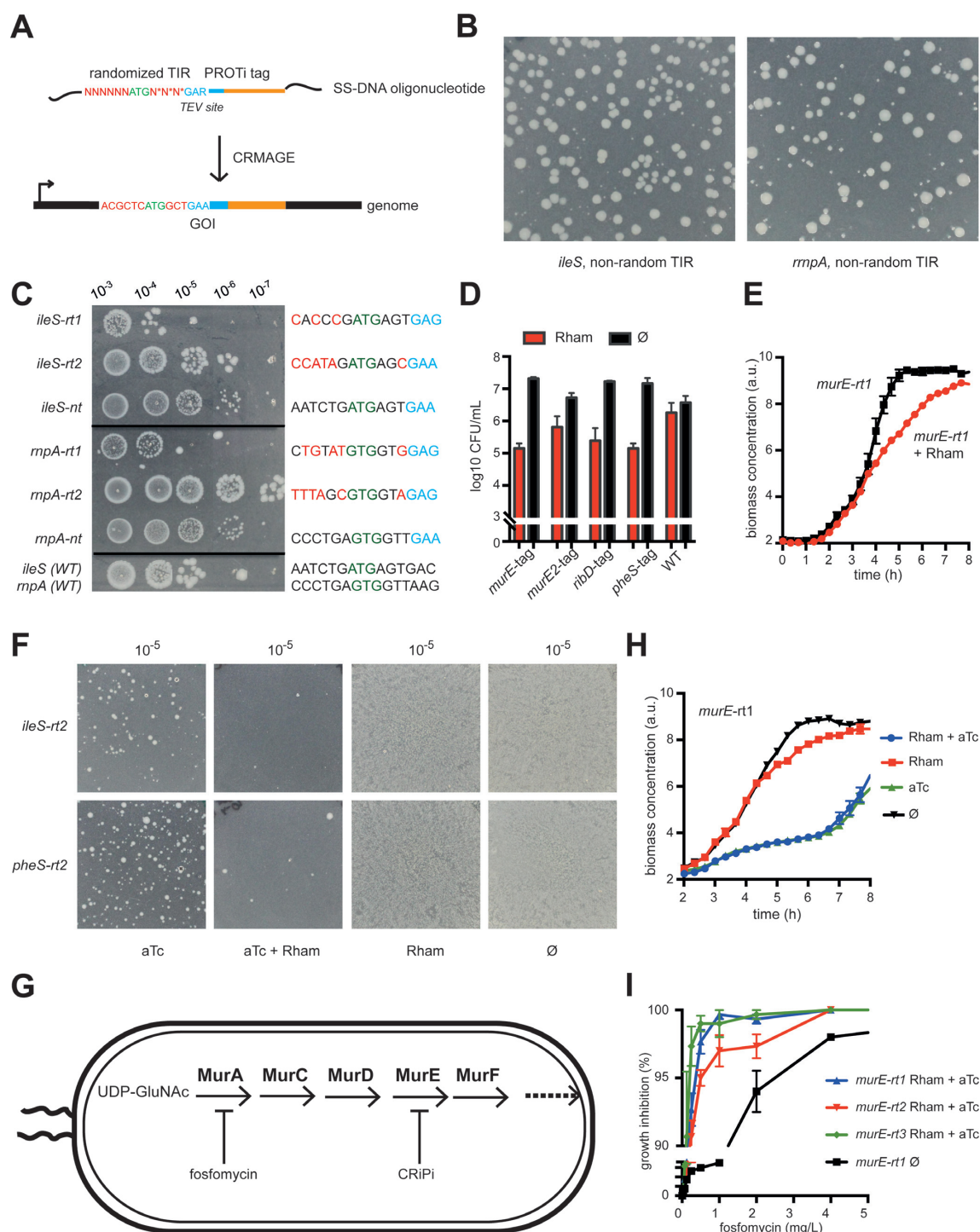


Figure 3. PROTi, CRiPi and TIR randomization can control protein levels and produce antibiotic hypersensitive bacteria. (A) Randomization of the translation initiation region (TIR, red font) in a ssDNA oligonucleotide enables CRMAGE-based insertion of the PROTi tag (orange) containing the TEV protease recognition site (blue) in genes of interest (GOI). (B) CRMAGE cultures plated directly after insertion of the PROTi tag in *ileS* (left) and *rnpA* (right) genes without TIR variation. Note the presence of both small and large colonies. The small colonies contained the PROTi tagged gene variants, as verified by colony PCR. (C) Drop tests of cells with *ileS* and *rnpA* tagged with and without TIR variation (rt: randomized TIR, nt: non-random TIR). TIR region sequences are indicated next to the different drop test lanes - red font indicates nucleotides different from the wild type sequence, blue font highlights the TEV protease recognition site and the start codon is highlighted in green. (D) Viability of control strain (WT with the PROTi plasmid) and PROTi variants tagged in the essential *E. coli* genes *murE*, *ribD* and *pheS* with and without rhamnose (Rham) induction. (E) Growth profile of *murE-rt1* cells carrying the PROTi system with and without Rham induction. (F) Agar plates illustrating the effect of *ileS*- and *pheS*-tagged cells with CRISPRi (aTc), PROTi (Rham) or CRiPi (aTc + Rham) induced after 4 h and plated after an additional 4 h of growth. Without inducers (Ø) growing bacteria completely cover the plate. (G) Illustration of the Mur enzymes involved in peptidoglycan biosynthesis pathway. (H) Growth profile of uninduced *murE-rt1* cells (Ø) or induced with Rham and/or aTc. (I) Fosfomycin sensitivity upon CRiPi induction in *murE*-tagged clones or no induction (Ø). Growth was tested in increasing concentrations of fosfomycin after 18 h incubation. All values are the averages of three biological replicates and bars show standard error.

ing the *in vivo* TEV protease activity, or by increasing the activity of the endogenous ClpA/P/S factors as shown previously in a similar system (29).

We were initially surprised by the almost complete lack of effect of PROTi in log phase cultures whereas subsequent plating resulted in several orders of magnitude reduction in growth for some of the PROTi targeted essential gene products. A similar observation was made recently in a high-throughput targeting of essential genes with CRISPRi in *B. subtilis* (11). There, it was suggested that (essential) protein levels are important for outgrowth from stationary phase, whereas maximal growth rate in log phase is less affected. It is possible that our CRISPRi system is more efficient than the one described for *Bacillus*, whereas the weaker effect of PROTi more resembles the *Bacillus* CRISPRi efficiency.

By inserting the sequence as a TIR variation library it is possible to create expression variants that can be screened with minimal polar effects, mimicking the natural gene expression level. Furthermore, some of the TIR variants, e.g. *rnpA*-rt1 and *rnpA*-rt1 (Supplementary Figure S2) or *murE*-rt1 and *murE*-rt2 (Figure 3H and Supplementary Figure S2) varied significantly in their sensitivity to CRISPRi, providing further support for the relationship between gene expression and CRISPRi efficiency (9). Thus, this type of multi-level reverse genetics tool may further expand the utility of the highly successful CRISPR/Cas system.

Bacterial antibiotic resistance is rapidly exhausting the number of available effective antimicrobial agents. Consequently, there is an urgent need to identify new target-specific inhibitors to develop antimicrobial compounds (28). With our combined CRiPi approach, insertion of a simple and inexpensive oligonucleotide enables subtle tuning of potential antibiotic targets on both the transcriptional and posttranslational level. Furthermore, the system represents a unique and versatile workflow that may enable future in-depth characterization of essential genes located in operons.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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